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Aldosterone interaction on sodium transport and chloride permeability: influence of epithelial structure

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Abstract

The effects of aldosterone on sodium transport and chloride permeability were investigated by electrophysiology in two structurally distinct epithelia used as models for the distal renal tubule: the A6 cell monolayer as compared with the amphibian skin epithelium (ASE). Short-circuit current (I_{sc}) and transepithelial conductance (G_t) were measured in A6 monolayers incubated overnight with(out) aldosterone. Cell and shunt conductances (G_{cell} and G_{sh}) were also determined, as well as the conductive nature of the chloride pathway. These parameters were correlated with sodium and chloride fluxes in A6 cells (J_{Na} and J_{Cl}) and compared with the data recorded across ASE (*Bufo marinus*). The existence of a cAMP-dependent chloride secretory pathway in A6 cells was also investigated upon exposition to arginine vasopressin (AVP) or oxytocin. When A6 monolayers were incubated with aldosterone, G_t significantly increased with respect to control preparations; this increase resulted solely from an increase in G_{cell}, and was reflected by a 3-fold increase in I_{sc}. There was a significant relationship between I_{sc} and G_{cell}, as well as between I_{sc} and J_{Na} in both control and aldosterone-stimulated preparations. The A6 clone used was devoid of cAMP-dependent chloride secretory activity and was unresponsive to AVP or oxytocin. Thus, comparison between ASE and A6 preparations revealed two major differences: unlike ASE, (i) aldosterone has no effect on G_{sh} and (ii) no conductive reabsorptive chloride pathway is operative in A6 monolayers tested. In addition, cobalt had no effect on electrical parameters of A6 monolayers. These observations show that difference in epithelial structure is reflected in terms of electrophysiological response to aldosterone, which suggests that cell heterogeneity could be a prerequisite for observing a conductive reabsorptive chloride pathway in aldosterone-responsive, sodium-transporting epithelia.

Keywords: A6 cell; Aldosterone; Sodium ion transport; Chloride permeability

1. Introduction

Investigation of the role played by aldosterone in the regulation of extracellular fluid volume has benefit greatly from electrophysiological studies conducted on high resistance amphibian skin epithelium (ASE), taken as a model for the cortical collecting duct (CCD) of the mammalian nephron. Aside from the action exerted by aldosterone on sodium transport across ASE [1], these techniques have made it possible to demonstrate that aldosterone increased chloride permeability of the same preparation [2]. This dual effect of aldosterone – on both sodium transport and chloride permeability – makes sense since expansion of the extracellular fluid volume requires net solute gain for the organism, i.e., that sodium transport be coupled to movement in the same direction of chloride in order to assure both electroneutrality and osmotic driving force [3].

If the stimulating action of aldosterone on transepithelial sodium transport is now well characterized [4], the issue of which structure(s) is (are) involved in transepithelial chloride permeability remains unsettled. In this regard, it must be emphasized that, in addition to their involvement in NaCl reabsorption, ASE and CCD have in common heterogeneity in terms of cell type. These epithelia contain a majority of principal (PR) cells, almost exclusively involved in active sodium reabsorption, and a small (5–10%) subpopulation of 'intercalated' (IC) or 'mitochondria-rich' (MR) cells, the physiological role of which is still debated [5,6]. Like IC cells of the CCD, the MR cells of ASE express high carbonic anhydrase activity and recent data point at their involvement in transepithelial

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chloride permeability [5,7] and acid-base regulation [8]. Although the MR cells do not seem to be involved in the stimulation of sodium transport by aldosterone, the hormonal effect on transepithelial chloride pathway seems indeed to be related to MR cell density [9]. The question was thus raised as to whether cellular heterogeneity of a target-epithelium is a prerequisite for the dual effect of aldosterone observed in ASE or, in other words, whether a specialized cell population is involved in the aldosterone-regulated chloride permeability.

In the present study, the aldosterone effect on both sodium transport and chloride permeability was investigated in another preparation, the A6 cell monolayer. A6 cells, originating from the distal portion of the renal tubule of *Xenopus laevis* [10], develop in culture as a flat polarized monolayer which is homogeneous in terms of cell type [11]. When grown at confluence on a permeable support, this monolayer behaves as a tight epithelium and transports sodium from the apical to the basolateral side, a property reflected by short-circuit current [11]. A6 cells possess receptors for aldosterone [12] and treatment with this hormone leads to increased Na^+/K^+-ATPase biosynthesis [13] as well as increased sodium transport [11]. Theoretically, these properties render A6 monolayers well suited for electrophysiological investigation of the effects of aldosterone on a homogeneous epithelium, as compared with those observed on ASE. In addition, there have been so far no systematic investigation of a putative conductive chloride reabsorption across A6 cell monolayers, in relation to their macroscopic electrical properties, and the influence exerted by aldosterone on this parameter is unknown.

We report here (i) that the structural difference between ASE and A6 cell monolayers is reflected in terms of electrophysiological response upon exposition to aldosterone; (ii) that, unlike ASE, no conductive chloride reabsorptive pathway appears to be operative in A6 cells and (iii) that, at variance with previous studies, the A6 clone used here has no adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent chloride secretory activity and is unresponsive to antidiuretic hormones.

Parts of this study have been presented in June 1993 at the XIIth International Congress of Nephrology, Jerusalem, Israel, and published in abstract form (Kidney Int. 46 (1994) 1246).

2. Methods

2.1. Cell culture

A6 cells were obtained from Dr. Johnson (Walter Reed Army Institute, Washington DC, USA) at passage 98. Passages 100–110 were used for experiments. The cells were seeded (at density approx. $(0.4–0.5) \cdot 10^5$ cells cm^{-2}) on plastic Petri dishes (55 cm^2) and placed in a humidified incubator aerated with 2% CO_2 in atmospheric air at 28° C. Growth medium was a mixture of 35% L-15 Leibovitz solution (Gibco), 35% F-12 Ham solution (Gibco), 20% H_2O, 8 mM $NaHCO_3$, 2 mM glutamine and 10% fetal bovine serum (Gibco); osmolarity was 240 mosmol l^{-1} and pH was 7.4. The medium was replaced three times a week. Confluence was achieved within 1 week, with cell density of $(4–5) \cdot 10^5$ cm^{-2}. For subculture, cells were dispersed by brief exposure to a 110 mM NaCl solution containing 0.25% trypsin (Boehringer) and 1 mM disodium ethylenediaminetetraacetate (Na_2EDTA). After rinsing and centrifugation, the pellet was resuspended and the cells were reseeded at high density (approx. $2 \cdot 10^5$ cm^{-2}) on filter paper culture inserts (Millicell HA: 0.45 μm pore size, Millipore PIHA 030050) of 4.2 cm^2 surface area. Serum-free medium was used once A6 cells had developed as confluent, tight monolayers, which usually took 2 weeks. Some preparations were incubated with aldosterone (10^{-7} M, Sigma) overnight prior to the experiments.

2.2. Electrical transepithelial measurements

Transepithelial potential difference (V) and short-circuit current (I_{sc}) were measured in an Ussing-type chamber equipped with sterile 3 M KCl-agar bridges, after transfer into modified Ringer's solution containing (in mM): NaCl 100, KCl 2.5, $CaCl_2$ 1, $MgCl_2$ 1, KH_2PO_4 1, $NaHCO_3$ 18, Hepes 5. The osmolarity of this solution was 230 mosmol l^{-1} and pH was 7.4. Transepithelial conductance (G_t) was calculated relying on Ohm's law; residual G_t during brief exposure of the apical cell surface to amiloride (10^{-4} M, Merck, Sharp and Dohme), so as to nullify cell conductance (G_{cell}), is considered as paracellular 'shunt' conductance (G_{sh}).

2.3. Sodium and chloride fluxes

For experiments meant to compare I_{sc} with net transepithelial sodium flux, the A6 monolayers were continuously short-circuited by an automatic voltage clamp device. ^{22}Na (New England Nuclear) was added to either apical or basolateral bathing solution (final concentration 0.8 μCi ml^{-1}), in experiments conducted on preparations from the same batch of A6 cells. Solutions on both sides were sampled after a 30-min equilibration period and again twice, after two 30-min flux periods. ^{22}Na was counted in a Berthold, model Mag 310 gamma counter. Chloride permeability of the A6 monolayers was evaluated in another set of preparations: as was the case for sodium, inward and outward chloride fluxes were measured in the short-circuited state, by means of ^{36}Cl (Amersham) counted by liquid scintillation spectrometry (Packard, model Tri-Carb 1500). The conductive nature of the chloride pathway was assessed in absence of sodium transport, resulting from treatment with amiloride (10^{-4} M) added to the

apical side, after replacing chloride on the basolateral side with NO_3 or gluconate.

2.4. Other electrophysiological investigations of A6 cells

In order to further characterize the A6 clone used in this study, and particularly to investigate the cAMP-dependent chloride secretory pathway previously documented in this cell line [14,15], the influence of oxytocin, an antidiuretic analogue of arginine vasopressin (AVP), was investigated. After incubation in a serum-free medium for 24 h, the confluent A6 monolayers were incubated with oxytocin or AVP ($2 \cdot 10^{-7}$ M on the serosal bath for both hormones), and electrical parameters recorded after short-term (0–20 min) and long-term (24 h) incubation. Unidirectional chloride fluxes were also recorded, as described above. Since divalent metal cations interfere readily and reversibly with conductive chloride flux in ASE [16], the effect of Co^{2+} on electrical parameters was also studied, after incubation of the preparations with $CoCl_2$ (1 mM, on the mucosal side).

2.5. Electrical measurements across amphibian skin

Some experiments were conducted on the abdominal skin of the toad, *Bufo marinus*, originating from the Dominican Republic, maintained on moist peat at 20° C and fed once weekly. After killing by double pithing, the abdominal skin was dissected free and matched pieces incubated overnight with(out) aldosterone ($5 \cdot 10^{-8}$ M) were mounted in Ussing-type chambers (incubation area: 3.14 cm^2) filled with aerated Ringer's fluid (NaCl 115 mM, $KHCO_3$ 2.5 mM, $CaCl_2$ 1 mM) for short-circuiting, according to Ussing and Zerahn [17].

2.6. Statistical analysis

Data are expressed as means ± S.E. with the number of measurements stated in parentheses. Significance of differences was assessed relying on Student's *t*-test.

3. Results

3.1. Effect of aldosterone on I_{sc} and G_t

When A6 monolayers were incubated overnight with aldosterone, together with a large increase in I_{sc} (by a factor 3 on average), G_t significantly increased, with respect to control preparations (Table 1). Assuming that amiloride interferes only with G_{cell} at least acutely [18], the aldosterone-induced increase in G_t was found to result solely from an increase in G_{cell}, as G_{sh} was not influenced by the hormone. As shown in Fig. 1, there was a significant relationship between I_{sc} and the amiloride-sensitive conductance (G_{cell}) of the preparations ($r = 0.67$, $P < 0.0001$).

Table 1
Influence of aldosterone on electrical parameters (I_{sc}: short-circuit current, G_t: transepithelial conductance, G_{cell}: cell conductance, G_{sh}: paracellular 'shunt' conductance) across A6 monolayers

	Control	Aldosterone	P
I_{sc} ($\mu A\ cm^{-2}$)	3.5 ±0.3 (31)	11.4 ±0.6 (20)	< 0.0001
G_t (mS cm^{-2})	0.20±0.01 (31)	0.26±0.01 (20)	0.0007
G_{cell} (mS cm^{-2})	0.07±0.01 (28)	0.12±0.01 (14)	0.0004
G_{sh} (mS cm^{-2})	0.13±0.01 (28)	0.14±0.01 (14)	N.S.

Aldosterone treatment consisted in exposing the preparations to the steroid hormone (10^{-7} M) overnight. The number of preparations studied is mentioned in parentheses. N.S., not significant.

3.2. Effect of aldosterone on J_{Na} and J_{Cl}

Unidirectional sodium fluxes were measured across A6 monolayers exposed or not to aldosterone (Table 2). The aldosterone-induced increase in I_{sc} was reflected by an increase in sodium influx ($J_{Na\ M \rightarrow S}$), while sodium efflux ($J_{Na\ S \rightarrow M}$) across the preparation was uninfluenced. As shown in Fig. 2, sodium influx across the preparation was related to I_{sc} in both control and aldosterone-stimulated preparations, confirming that I_{sc} may be reliably used as an indicator of sodium transport. Noteworthy, the calculated value of sodium influx at $I_{sc} = 0$ (19 pEq/cm^2 per s) corresponded closely to the measured sodium efflux (Table 2). Furthermore, net transepithelial sodium flux (i.e.,the difference between $J_{Na\ M \rightarrow S}$ and $J_{Na\ S \rightarrow M}$ as appears in Table 2) was of the same magnitude as I_{sc} (in the case of a monovalent ion, an I_{sc} value of 1 $\mu A\ cm^{-2}$ reflects a net flux of 10.3 pEq/cm^2 per s).

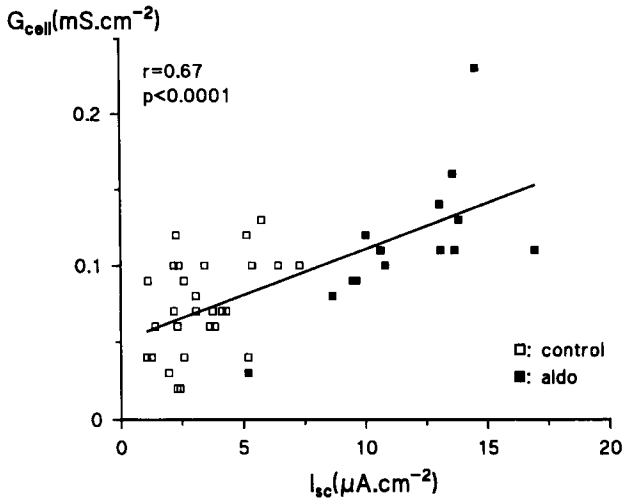

Fig. 1. Relationship between short-circuit current (I_{sc}) and amiloride-sensitive cell conductance (G_{cell}) of A6 monolayers incubated overnight with ($n = 14$) or without ($n = 28$) aldosterone (10^{-7} M). The regression equation reads: $y = 6.06 \cdot 10^{-3}\ x + 0.05$.

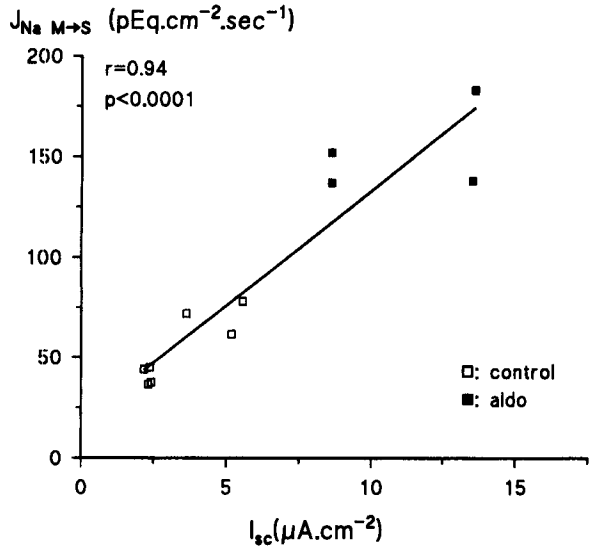

Fig. 2. Relationship between short-circuit current (I_{sc}) and sodium influ: ($J_{Na\ M\to S}$) across A6 monolayers incubated overnight with ($n=4$) or without ($n=7$) aldosterone (10^{-7} M). The regression equation reads: $y=11.4x+18.9$.

By contrast, inward ($J_{Cl\ M\to S}$) and outward ($J_{Cl\ S\to M}$) chloride fluxes across A6 monolayers were of similar magnitude and were not influenced by aldosterone (Table 2).

Table 2
Unidirectional sodium and chloride fluxes across A6 monolayers incubated overnight with or without aldosterone (10^{-7} M)

	Control	Aldosterone	P
$J_{Na\ M\to S}$	53±6 (7)	153±11 (4)	< 0.0001
$J_{Na\ S\to M}$	17±2 (10)	16± 2 (5)	N.S.
$J_{Cl\ M\to S}$	29±3 (7)	32± 7 (7)	N.S.
$J_{Cl\ S\to M}$	28±5 (7)	20± 4 (4)	N.S.

M → S (mucosal to serosal) indicates influx and S → M (serosal to mucosal) indicates efflux across the preparation. Ion fluxes are expressed in pEq/cm^2 per s. Number of preparations tested in parentheses. N.S., not significant.

Table 3
Effect of amiloride (10^{-4} M, added on the mucosal bath) on the electrical parameters recorded across A6 monolayers incubated overnight with or without aldosterone (10^{-7} M)

	Control	Amiloride	P
I_{sc} (μA cm^{-2})			
Control (11)	3.2±0.5	0.05±0.01	< 0.0001
Aldo (8)	11.0±0.5	0.33±0.17	< 0.0001
G_t (mS cm^{-2})			
Control (11)	0.23±0.03	0.15±0.02	0.02
Aldo (8)	0.30±0.3	0.14±0.02	< 0.001

The maximal effect was always recorded within 15 min following amiloride exposure. Number of experiments in parentheses.

3.3. Is there any conductive counterpart to J_{Cl} in A6 monolayers?

Effect of amiloride

When amiloride was added to the solution bathing the apical surface of A6 monolayers, I_{sc} was promptly and almost totally inhibited, irrespective of previous incubation with aldosterone (percentage of inhibition: 98 ± 0.5 in control vs. 97 ± 1 in aldosterone-treated preparations). A significant decrease in terms of G_t was also observed, both in control and aldosterone-stimulated preparations (Table 3).

Chloride reabsorption

In order to investigate specifically a chloride reabsorptive pathway, with particular attention paid to a conductive

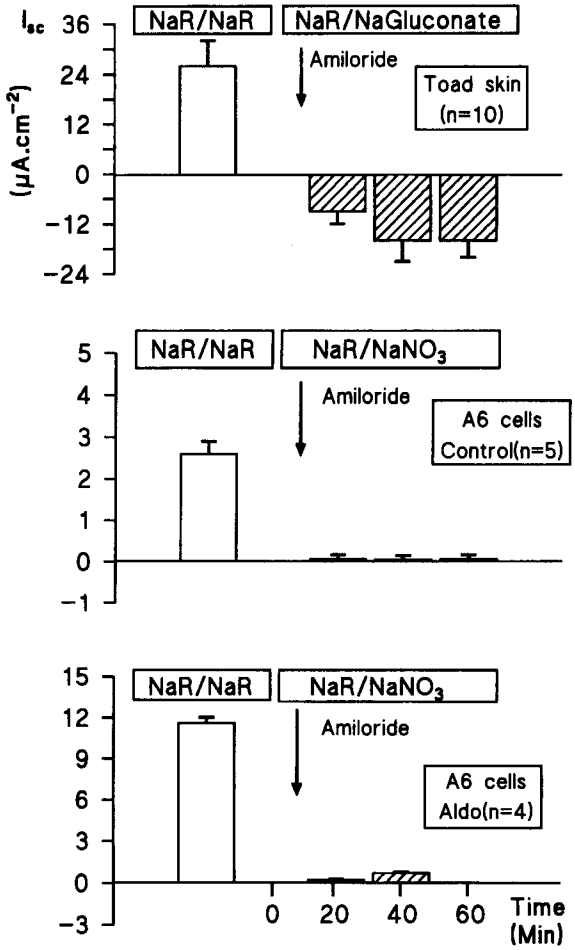

Fig. 3. Short-circuit current (I_{sc}) in control conditions (incubation in standard Ringer's on both sides of the preparation) and upon imposing a chloride gradient by replacing chloride with an impermeant anion (NO_3 or gluconate) in the basolateral bathing solution, while sodium transport is inhibited with amiloride (10^{-4} M, added to the apical bathing solution): behaviour of ASE (*Bufo marinus*) compared to that of A6 monolayers. In contrast with what is observed in ASE, there is no evidence for a conductive counterpart to J_{Cl} in control or aldosterone-stimulated A6 monolayers. Note that, in ASE, either gluconate or NO_3 could be used as basolateral impermeant anion without affecting the magnitude of the conductive J_{Cl} inward.

counterpart to J_{Cl}, sodium transport was inhibited by amiloride (in these conditions, as $G_{cell} = 0$, $G_t = G_{sh}$) and a chloride gradient was created across A6 monolayers by replacing chloride in the basolateral solution with NO_3 (replacement of basolateral chloride with gluconate was followed by electrical instability; therefore, NO_3 was used routinely as unpermeant anion). This manipulation did not lead to the development of an electrical potential difference such as was observed when ASE was handled similarly [7]. In these conditions indeed, a 'reversed' (negative) short-circuit current was obtained in ASE; this stands in strong contrast with what was observed in A6 monolayers, where I_{sc} fell to zero despite the chemical driving force for chloride influx in absence of sodium transport (Fig. 3). Noteworthy, $J_{Cl\ M \to S}$ across A6 monolayers incubated in the presence of a chemical gradient for chloride inward was similar in magnitude (21 ± 4 pEq/cm^2 per s) to what was observed when preparations were incubated with Ringer's on both sides (Table 2). In addition, creation of an electrical gradient across the preparation by clamping the serosal side at +20 mV failed to increase significantly $J_{Cl\ M \to S}$ (18 ± 1 vs. 25 ± 4 pEq/cm^2 per s, control vs. clamp, $n = 4$).

Chloride secretion

In view of previous studies demonstrating the existence of a cAMP-stimulated chloride secretory pathway in A6 cells, our preparations were tested with oxytocin, an antidiuretic analogue of AVP. At baseline, in control or aldosterone-treated preparations, no net chloride efflux was observed under short-circuit conditions (Table 2). Addition of oxytocin to the serosal side of the preparations led to a

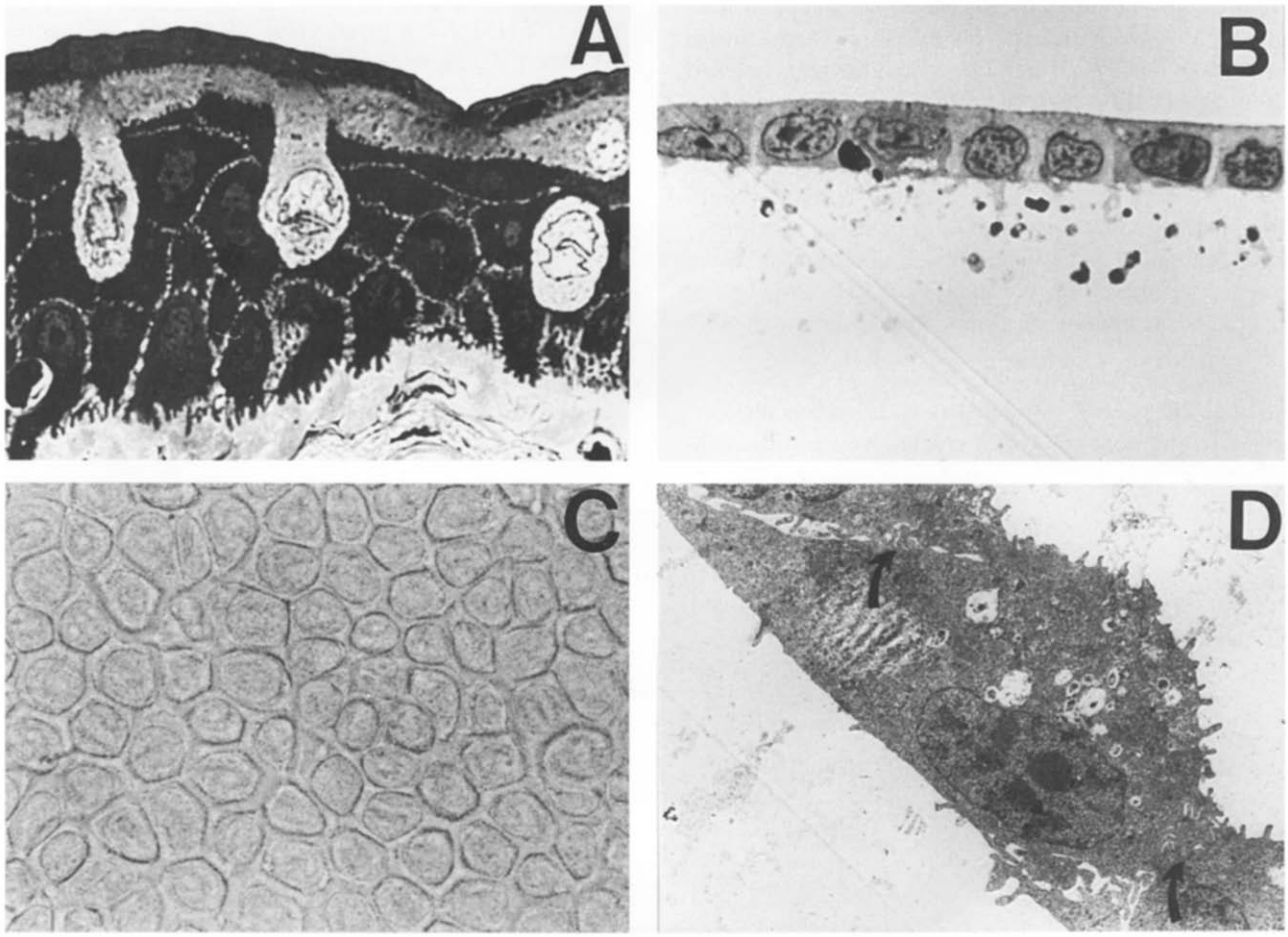

Fig. 4. Morphology of ASE and A6 cell monolayers. (A and B) Semi-thin (1 μm), toluidine blue-stained section of Epon-embedded ASE (A) as compared with an A6 monolayer grown on semi-permeable filter (B). On these sections perpendicular to the epithelial surface, the structural differences between the two preparations are obvious: the ASE is a multilayered heterogeneous epithelium (next to a majority of PR cells, which appear dark and are interconnected by desmosomes and gap junctions, it contains 5–10% of MR cells, which appear clear, show a flask-like shape and are not connected by gap junctions to the PR cells), while the A6 monolayer displays cell homogeneity. (×1250). (C) Apical view of an A6 cell monolayer grown at confluence on permeable filter. The A6 cells form a clear homogeneous population of small polygonal cells. (×315). (D) Ultra-thin section of A6 cells grown on permeable filter. Two weeks after seeding, the cells are interconnected by junctional complexes (arrows) to form a tight monolayer. All the cells present a similar morphology, characterized by a cuboidal shape with short and rare apical microvilli. The monolayers were fixed for 2 h at 4° C in 2.5% glutaraldehyde and postfixed with osmic acid; they were dehydrated in a graded series of ethanol and embedded in Epon LX112 (Electron Microscopy Sciences). Ultra-thin sections (60–100 nm) were poststained with uranyl acetate and lead citrate and viewed under a Philips electron microscope. (×8000).

Table 4
Short-term (20 min) influence of oxytocin ($2 \cdot 10^{-7}$ M, serosal bath) on electrical parameters and unidirectional chloride fluxes across A6 monolayers

	Control (11)	Oxytocin (9)	P
I_{sc} ($\mu A\ cm^{-2}$)	3.2 ±0.5	7.2 ±0.9	< 0.001
G_t (mS cm^{-2})	0.23±0.03	0.35±0.03	< 0.001
G_{cell} (mS cm^{-2})	0.08±0.02	0.15±0.02	0.01
G_{sh} (mS cm^{-2})	0.15±0.02	0.20±0.03	N.S.
$J_{Cl\ M \rightarrow S}$ (pEq/cm^2 per s)	43±7	38±8	N.S.
$J_{Cl\ S \rightarrow M}$ (pEq/cm^2 per s)	43±3	41±7	N.S.

The preparations were incubated in a serum-free medium 24 h before investigation. Number of preparations tested in parentheses. N.S., not significant.

significant increase in G_t, probably reflecting an increased sodium-transporting activity (as attested by an increase both in I_{sc} and G_{cell}), while G_{sh} was similar (Table 4). Accordingly, the amiloride-induced inhibition of I_{sc} was not significantly modified by oxytocin (percentage of inhibition: 98 ± 1 in control vs. 94 ± 2 in oxytocin-treated preparations). Finally, no significant modifications in terms of chloride flux were recorded during oxytocin exposition (Table 4). The effects of oxytocin were maximal within 20 min following exposure; no further changes were observed after long-term (24 h) incubation with the hormone. Similar observations – no significant modifications in terms of G_{sh} and percentage of I_{sc} inhibition by amiloride – were obtained after short-term (15 min) and long-term (24 h) incubation of A6 monolayers with AVP (data not shown).

Effect of divalent metal cations

Among others, cobalt cation has been shown to interfere readily and reversibly with conductive chloride pathway and shunt conductance of ASE, when added to the mucosal incubation medium [16]. At variance with these observations, there was no such modification of G_{sh} in A6 monolayers incubated with Co^{2+} (G_{sh}: 0.26 ± 0.08 vs. 0.26 ± 0.08 mS cm^{-2}, n = 9).

3.4. Influence of epithelial structure on the electrophysiological effects of aldosterone

The relationship between changes in G_t vs. those in sodium-transporting activity (monitored by I_{sc}) as a function of aldosterone treatment was investigated in A6 monolayers as in ASE. In NaCl-reabsorbing epithelia, G_t exceeds the conductance of principal cells (G_{cell}) which carry out most of sodium transport by the tissue and which can be impaled by microelectrodes. Since G_{cell} depends mainly on sodium conductance of the apical border of the principal cells [18], for a given value of G_{sh}, G_t will increase whenever I_{sc} increases. On the other hand, G_{sh} depends critically on chloride [19]. Thus, the slope $\Delta G_t/\Delta I_{sc}$ should provide an indication about a possible effect on G_{sh} of agents influencing I_{sc}. When homogeneous (A6) or heterogeneous (ASE) preparations in terms of cell type (Fig. 4) are examined at the light of this formal approach, this ratio is much larger in ASE than in A6 monolayers exposed to aldosterone ($\Delta G_t/\Delta I_{sc}$: 0.034 ± 0.006 vs. 0.009 ± 0.003 respectively, $P < 0.0001$) (Fig. 5). This difference depends of the presence of chloride on the apical side of ASE; indeed, when ASE was incubated in absence of chloride in the apical bathing solution, the ratio (0.010 ± 0.003) became similar to that obtained for A6 cells. From the

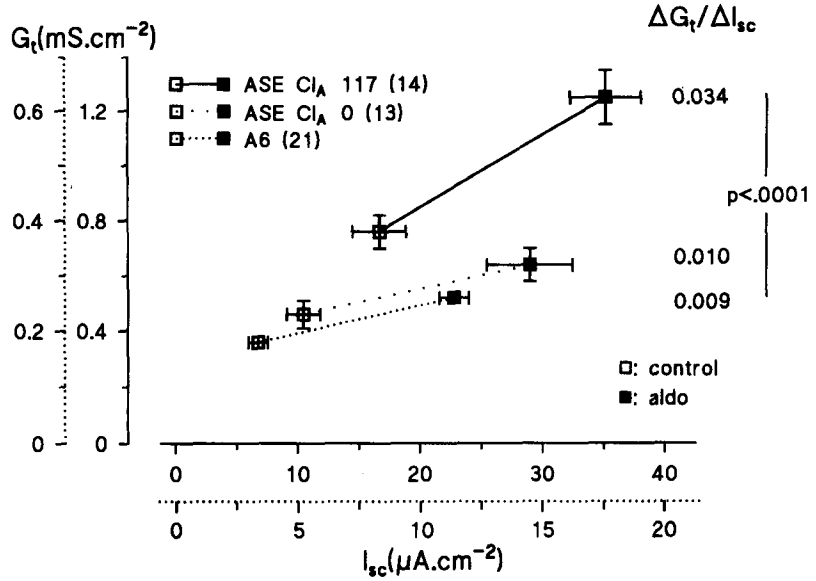

Fig. 5. Effect of overnight incubation with aldosterone on the relationship between short-circuit current (I_{sc}) and transepithelial conductance (G_t) in three different amphibian preparations (number of preparations in parentheses): amphibian skin epithelium incubated in standard Ringer's solution (ASE, Cl_A-117), the same incubated in absence of chloride (replaced by gluconate) in the apical bath (ASE, Cl_A-0) and A6 monolayers (A6). For each preparation, the slope ($\Delta G_t/\Delta I_{sc}$) is meant to indicate an effect of aldosterone on transepithelial chloride movement, reflected in shunt conductance (G_{sh}). Given the lower magnitude, relative to ASE, of the parameters recorded across A6 monolayers, both scales are reduced by half for the latter preparations.

foregoing, it can be concluded that G_{sh} – reflecting chloride conductance -- was influenced by aldosterone in ASE but not in A6 cells, which provides another indication that there is a qualitative electrophysiological difference between A6 and ASE preparations.

4. Discussion

The present study shows that there are fundamental differences between A6 monolayers and ASE in terms of electrophysiological properties and response to aldosterone. In contrast with what is observed in ASE [2,7], the aldosterone-induced increase in A6 monolayer conductance results solely from an increase in G_{cell}, the latter being expected in view of increased sodium transport (monitored by I_{sc} and J_{Na}). Aldosterone is devoid of any effect on G_{sh} and J_{Cl} in A6 cell monolayers; there is no evidence for a conductive chloride pathway in this preparation, either in baseline conditions or after treatment with aldosterone or antidiuretic hormones whose main action is to raise intracellular cAMP.

An increase in the rate of transepithelial sodium transport is the characteristic response of the distal part of the nephron to mineralocorticoid hormones [20]. The existence of a positive natriferic response to aldosterone in A6 epithelia – originating from the distal tubule of *Xenopus laevis* kidney – is well established [11,21,22] and can also be observed in our A6 clone. As shown in Tables 1 and 2, there is good agreement between electrical and sodium flux measurements, which indicates clearly that sodium influx is reliably reflected by I_{sc} both in control and in aldosterone-stimulated A6 cell preparations (Fig. 2). When compared with the initial observations of Perkins and Handler [11], it appears that baseline I_{sc} and G_t are slightly higher in our preparations, the relative magnitude of I_{sc} stimulation by aldosterone (3-fold) being very similar. On the other hand, the significant increase in G_t (solely due to an increase in G_{cell}) observed in our preparations incubated overnight with aldosterone was not reported by these authors. Although both studies were performed 2 weeks after seeding the cells, at a time when electrical parameters are reported to be stable [22], Perkins and Handler [11] used A6 cells of lower passage number; therefore, the discrepancy could possibly be related to some maturation effect following incubation with aldosterone, with the increase in G_{cell} matched by a decrease in G_{sh} such that G_t was unchanged (J. Crabbé, unpublished observation). Other studies on A6 monolayers have shown considerable variations in terms of basal I_{sc} (varying from 0.3 to 23 $\mu A\ cm^{-2}$, as reviewed in [22]) and responsiveness to aldosterone [13], possibly reflecting variations in age of culture, experimental conditions or clonal selection [13,22]. When the effects of aldosterone were studied in A6 cell monolayers in open-circuit conditions, stimulation of active sodium transport was correlated with an increase in paracellular ion flux [21]. Since short-circuiting electrically uncouples the paracellular pathway from active transcellular sodium transport, this approach is required to assess G_{sh}; in these conditions, as shown here, the aldosterone-induced increase in sodium transport was solely associated with an increase in G_{cell}, G_{sh} remaining unchanged.

In their original report, Perkins and Handler noted that inhibition of I_{sc} by amiloride was incomplete in A6 monolayers exposed to cAMP [11]. This amiloride-insensitive component is activated by either AVP or forskolin and reflects net transepithelial chloride secretion across the preparation [15,23]. This chloride secretion appears to be channel-mediated, since at least two types of chloride channels (single channel conductance of 3 and 8 pS) have been identified in the apical membrane of confluent A6 cells [24]; another channel of much larger conductance (400 pS) was observed only in subconfluent preparations [25]. Evidence was also provided for the presence of a Na-K-2Cl-coupled transporter in the basolateral plasma membrane of A6 cells, which also plays an important role in net basal to apical chloride flux [15]. At variance with these data, several lines of evidence suggest that this chloride secretory pathway is not operative in the A6 clone used here. First, at baseline, our clone is characterized by a 98% inhibition of I_{sc} by amiloride, a greater value than those (75 to 95%) previously mentioned [11,14,15]. Second, exposure of our A6 monolayers to either AVP or oxytocin is not followed by an increase in the amiloride-insensitive I_{sc}, since a 96% inhibition of I_{sc} by amiloride is still observed. This stands in contrast with other studies in which similar exposure to antidiuretic hormones was followed by a drop of the amiloride-induced inhibition of I_{sc} to values ranging from 40 to 71% [14,15,26,27]. Third, G_{sh} is not influenced by exposition to oxytocin or AVP (Table 4). Again, possible explanations for these differences between A6 preparations include variations in terms of cell aging, tissue culture conditions or clonal selection [22]. On the other hand, although A6 monolayers are supposed to be constituted by a single cell type, morphological heterogeneity has been observed within some A6 subclones grown on filters [28], so that apical sodium and chloride conductance could theoretically be located in various cell types [27]. In this respect, as shown in Fig. 4, a strict morphological homogeneity characterizes the A6 clone used here; this homogeneity has been confirmed by analysis of various immunocytochemical properties (positive immunostaining for Na^+/K^+-ATPase and absence of immunoreactivity for both type II carbonic anhydrase and band 3) (O. Devuyst and B. Lyoussi, unpublished observations).

Although active chloride secretion has been documented in rabbit CCD under certain conditions, direct evidence for chloride secretion at the distal tubule is limited and the relative importance of a chloride secretory pathway at this level of the nephron is weak when compared with reabsorptive processes [29]. In this regard, little

attention has been paid so far to investigate a putative chloride reabsorption across A6 monolayers. Fidelman and Watlington suggested the existence of chloride exchange diffusion in A6 monolayers [21], but their study was performed under open-circuit conditions – which renders difficult an adequate evaluation of G_{sh}. Recently, Verrey et al. showed that vasotocin could induce a large increase in chloride reabsorption in aldosterone-treated A6 monolayers [27]. Again, these findings were obtained only in open-circuit conditions; under short-circuit conditions, the vasotocin-induced increase in chloride permeability led to chloride secretion across the preparation. In addition, the possibility of morphological heterogeneity among the A6 clone used in the latter study was mentioned [27,28]. We have investigated the chloride permeability of A6 monolayers by measuring inward and outward chloride flux in the short-circuited state. As shown in Table 2, these fluxes are symmetric and unresponsive to aldosterone; similarly, oxytocin was devoid of any effect on this parameter (Table 4). Because chloride flux across most amphibian epithelia is passive, the existence of a conductive chloride reabsorptive pathway was examined by combining inhibition of sodium transport and establishment of a chloride gradient across the preparation. In these conditions, inhibition of I_{sc} by amiloride was virtually complete, both in baseline conditions and after incubation with aldosterone; at variance with ASE, there was no conductive component to J_{Cl} inward in control or aldosterone-stimulated A6 preparations (Fig. 3).

These data have to be considered when addressing the issue of the anatomical counterpart to the electrophysiological behaviour of two aldosterone-responsive epithelia used as models for the distal renal tubule. Fundamentally, we found two major differences between A6 cell monolayer and ASE preparations. First, whereas hormonal stimulation of G_t largely exceeded that of I_{sc} in ASE incubated in Ringer's, no such G_t increase was observed in A6 monolayers incubated similarly. This additional G_t increase observed in ASE critically depends on the presence of chloride in the apical bathing solution. Of interest is that the electrophysiological response to aldosterone of ASE and A6 monolayers was identical when chloride was removed from the apical surface (Fig. 5). Second, a conductive chloride influx could be observed across ASE, the magnitude of which was increased by aldosterone [9]; this stands in contrast with the behaviour of A6 cell preparations incubated in the same conditions, as shown here (Fig. 3). A third difference came when examining the effect of cobalt on A6 monolayers. It has been suggested that divalent heavy metal cations, which rapidly and reversibly reduce chloride-related tissue conductance, might be valuable for characterization of transepithelial conductive chloride transport [30]. In contrast with ASE, in which both transepithelial chloride influx and short-circuit current were significantly reduced by cobalt added to the mucosal side [16], no such inhibition was observed in A6 cells.

It is tempting to consider that these differences are related to the structure of the target epithelium (Fig. 4). As mentioned above, the A6 monolayers are supposed to be homogeneous in terms of cell type, with properties of PR cells carrying out active sodium transport. In contrast, ASE contains both PR cells and MR cells, the latter being considered as analogous to the IC cells of the CCD [5]. The PR cells of ASE lack apical chloride conductance [18]; therefore, the latter property might be related to the other cell population, namely the MR cells. We have shown that, aside from stimulating sodium transport, aldosterone induced a significant increase in chloride conductance of ASE; there are indications that the latter involves the MR cells, and that aldosterone influences the functional state of these cells [2,9]. Voûte and Meier [31] were first to stress the relationship between the MR cells in ASE and the magnitude of chloride conductance; their observation has received support from vibrating-probe data [32], cell volume measurements [33] and immunocytochemical studies [34]. However, the exact role played by the MR cells in the chloride pathway remains controversial [6]; the same could be said about the CCD, in which a paracellular chloride conductance has been reported [35], as well as the presence of apical chloride channels in IC [36].

At any rate, our data demonstrate that no conductive chloride permeability could be revealed in the A6 clone used. Since this preparation is characterized by functional and morphological homogeneity, these observations suggest that cells distinct from PR cells are a prerequisite for observing a reabsorptive, conductive and aldosterone-sensitive transepithelial chloride pathway. Thus, another indication is provided that a cell population distinct from PR cells is involved in the regulation of chloride permeability of aldosterone-sensitive sodium-transporting epithelia.

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References

[1] Crabbé, J. (1964) Endocrinology 75, 809–811.
[2] Beauwens, R., Beaujean, V., Zizi, M., Rentmeesters, M. and Crabbé, J. (1986) Pflügers Arch. 407, 620–624.

[3] Devuyst, O. and Crabbé, J. (1993) Nephrol. Dial. Transplant. 8, 379–380.
[4] Garty, H. (1986) J. Membr. Biol. 90, 193–205.
[5] Larsen, E.H. (1991) Physiol. Rev. 71, 235–283.
[6] Nagel, W. and Dörge, A. (1990) Pflügers Arch. 416, 53–61.
[7] Devuyst, O., Beaujean, V. and Crabbé, J. (1991a) Pflügers Arch. 417, 577–581.
[8] Ehrenfeld, J., Lacoste, I. and Harvey, B.J. (1989) Pflügers Arch. 414, 59–67.
[9] Devuyst, O., Beaujean, V. and Crabbé, J. (1991b) Biochim. Biophys. Acta 1066, 268–270.
[10] Rafferty, K.A. (1969) in Biology of Amphibian Tumors (Mizell, M., ed.), pp. 52–81, Springer-Verlag, Berlin.
[11] Perkins, F.M. and Handler, J.S. (1981) Am. J. Physiol. 241, C154–C159.
[12] Watlington, C.O., Perkins, F.M., Munson, P.J. and Handler, J.S. (1982) Am. J. Physiol. 242, F610–F619.
[13] Verrey, F., Schaerer, E., Zoerkler, P., Paccolat, M.P., Geering, K., Kraehenbuhl, J.P. and Rossier, B.C. (1987) J. Cell Biol. 104, 1231–1237.
[14] Chalfant, M.L., Coupaye-Gerard, B. and Kleyman, T.R. (1993) Am. J. Physiol. 264, C1480–C1488.
[15] Yanase, M. and Handler, J.S. (1986) Am. J. Physiol. 251, C810–C814.
[16] Beaujean, V. and Crabbé, J. (1992) Biochim. Biophys. Acta 1104, 174–178.
[17] Ussing, H.H. and Zerahn, K. (1951) Acta Physiol. Scand. 23, 110–127.
[18] Nagel, W. (1977) J. Physiol. 269, 777–796.
[19] Linderholm, H. (1953) Acta Physiol. Scand. 28, 211–217.
[20] Koeppen, B.M. and Stanton, B.A. (1992) in The Kidney: Physiology and Pathophysiology (Seldin, D.W. and Giebisch, G., eds.), pp. 2003–2039, Raven Press, New York.
[21] Fidelman, M.L. and Watlington, C.O. (1987) Biochim. Biophys. Acta 931, 205–214.
[22] Wills, N.K. and Millinoff, L.P. (1990) Pflügers Arch. 416, 481–492.
[23] Preston, A.S., Muller J. and Handler, J.S. (1988) Am. J. Physiol. 255, C661–C666.
[24] Marunaka, Y. and Eaton, D.C. (1990) Am. J. Physiol. 258, C352–C368.
[25] Nelson, D.J., Tang, J.M. and Palmer, L.G. (1984) J. Membr. Biol. 80, 81–89.
[26] Bindels, R.J.M., Schafer J.A. and Reif, M.C. (1988) Biochim. Biophys. Acta 972, 320–330.
[27] Verrey, F. (1994) J. Membr. Biol. 138, 65–76.
[28] Verrey, F., Digicaylioglu, M. and Bolliger, U. (1993) J. Membr. Biol. 133, 213–226.
[29] Simmons, N.L. (1993) Exp. Physiol. 78, 117–137.
[30] Nagel, W., Natochin, Y. and Crabbé, J. (1988) Pflügers Arch. 411, 540–545.
[31] Voûte, C.L. and Meier, W. (1978) J. Membr. Biol. 40, 151–165.
[32] Katz, U. and Scheffey, C. (1986) Biochim. Biophys. Acta 861, 480–482.
[33] Larsen, E.H., Ussing, H.H. and Spring, K.R. (1987) J. Membr. Biol. 99, 25–40.
[34] Devuyst, O., Rott, R., Denef, J.F., Crabbé, J. and Katz, U. (1993) Biol. Cell 78, 217–221.
[35] Schuster, V.L. and Stokes, J.B. (1987) Am. J. Physiol. 253, F203–F212.
[36] Light, D.B., Schwiebert, E.M., Fejes-Toth, G., Naray-Fejes-Toth, A., Karlson, K.H., McCann, F.V. and Stanton, B.A. (1990) Am. J. Physiol. 258, F273–F280.